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Rapid Communication

Regulation of MCP-1 gene transcription by Smads and HIV-1 Tat in human glial cells

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Abstract

Expression of several cytokines involved in signal transduction such as TGFβ-1 and the inflammatory chemokines including MCP-1 is elevated during the course of AIDS progression. The enhancement of these cellular proteins in astrocytic cells is mediated, at least in part, by HIV-1 Tat protein. Here, we investigate the possible regulation of MCP-1 transcription by Tat and the Smad family of transcription factors whose activities are induced by the TGFβ-1 pathway. Results from transfection studies revealed that Smad-3 stimulates basal and Tat-mediated transcription of MCP-1 in human astrocytic cells. Smad-4, on the other hand, had no effect on the basal activity of the MCP-1 promoter, but showed the ability to decrease both Smad-3 and Tat-induced transcription of the MCP promoter. Results from protein-binding studies revealed the ability of both Smad-3 and Smad-4 to associate with the region of Tat spanning residues 1–40. Examination of the transcriptional activity of the various domains of Smad including MH1, at the N-terminus, and MH2, at the C-terminus of the protein indicated that neither MH1 or MH2 alone positively cooperate with Tat in modulating MCP-1 transcription. However, ectopic expression of MH1 and, more notably, MH2 severely suppressed transcriptional activation of MCP-1 by Tat in astrocytic cells. Binding studies revealed that similar to the full-length Smad protein, both MH1 and MH2 associate with Tat protein and that the residues between 1 and 40 of Tat are important for their interaction. These observations reveal a novel mechanism for Tat-mediated transcriptional activation via TGFβ signaling pathway and provide evidence for regulation of MCP-1 gene transcription by this signaling pathway in human astrocytic cells.

A variety of direct and indirect stimuli such as viral infection, trauma, and autoimmune disorders can result in the activation and directed migration of leukocytes into the area of tissue injury. One of the major complications of HIV-1 infection is the syndrome of cognitive and motor dysfunction, so-called HIV-1-associated dementia (HAD) (Lipton and Gendelman, 1995). The neuropathology associated with HAD, termed encephalitis, is characterized by infiltration of immune cells, particularly monocytic cells such as macrophages and microglia, by myelin pallor, and by reactive astrocytosis (Budka et al., 1987; Nielson et al., 1994; Sharer and Kapila, 1985). While the mechanism responsible for monocytic infiltration of the brain remains elusive, there have been several reports suggesting that enhanced expression of several cytokines and chemokines by the infected monocytic cells as well as astrocytes may be involved in the recruitment of the inflammatory cells. For example, enhancement in the level of transforming growth factor, TGFβ, a cytokine with a potent chemotactic activity for monocytes, has been observed in HIV-1-infected monocytic macrophages both in cell culture and in brain tissue of AIDS patients (Wahl et al., 1987, 1991). Accordingly, our results show that HIV-1 regulatory protein, Tat, enhances the promoter activity of TGFβ in human astrocytic cells (Cupp et al., 1993; Rasty et al., 1996; Sawaya et al., 1998). On the other hand, several reports link HIV-1 infection of macrophages to an increased level of several monocyte chemoattractant chemokines such as Rantes, MIP1α, and MIP1β (Canque et al., 1996; Mengozzi et al., 1999;

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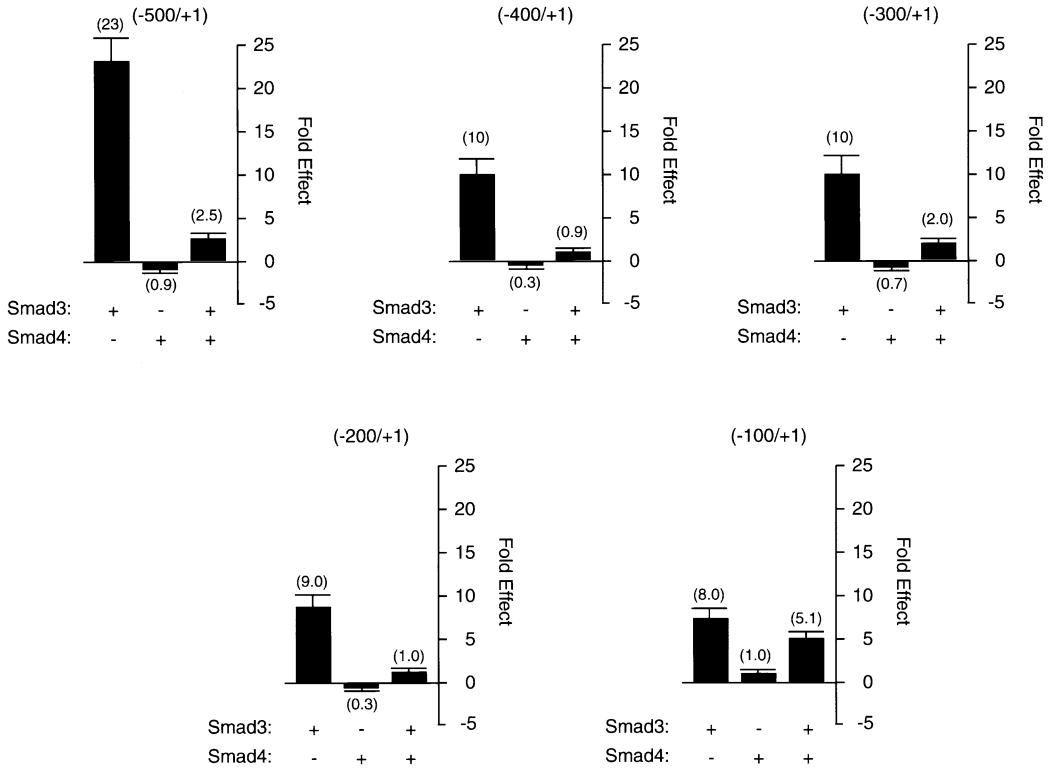

Fig. 1. Regulation of the MCP-1 promoter by Smad-3 and Smad-4. A series of DNA fragments corresponding to the MCP-1 promoter region spanning nucleotides −500 to +1, −400 to +1, −300 to +1, −200 to +1, and −100 to +1 were created by gene amplification using specific DNA primers. The amplified DNAs were placed at the 5′ region of the CAT reporter gene and after verification by direct sequencing, each reporter construct was used in the transfection assay. In the transfection study, the human astrocytic glial cell line, U-87MG (ATCC), was maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum (Gibco-BRL, Rockville, MD) and supplemented with antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G). Cells (5×10^5) cultured on 60-mm plates and grown overnight were transfected by the calcium phosphate precipitation method as described by Gallia et al. (1999) with 5.0 μg of the reporter DNA either alone or together with 5.0 μg of a plasmid expressing Smad-3 and Smad-4. The amount of DNA used for transfection was normalized with pCDNA-3 plasmid. Cell extracts were prepared 48 h after transfection and CAT assay was performed. The bar graphs demonstrate an average fold effect of Smad-3, Smad-4, and Smad-3 plus Smad-4 on the basal MCP-1 promoter activity from three independent experiments.

Schmidtmayerova et al., 1996). Furthermore, induction of the chemoattractant protein 1 (MCP-1), one of the most potent monocyte chemoattractants which are commonly induced in astrocytes, has been reported during the course of AIDS dementia (Conant et al., 1998). Similar to TGFβ, the elevation of MCP-1 expression in astrocytes seems to be mediated by the Tat protein (Conant et al., 1998; Lim and Garzino-Demo, 2000).

Tat is a small early viral protein whose expression is essential for the transcription of the HIV-1 genome in the infected cells. To exert its activity, Tat interacts with the TAR RNA sequence located in the leader of the viral transcript where it associates with several cellular proteins which are involved in the process of transcription initiation and elongation (Herrmann and Rice, 1995). In addition, several reports have established the ability of Tat to stimulate transcription of TAR-negative cellular genes including TNFα, TGFβ, MCP-1, and IL-1 through alternative mechanisms that involve its association with several DNA-binding proteins (Conant et al., 1998; Cupp et al., 1993; Glass et al., 1993; Nottet et al., 1995; Sawaya et al., 1998; Zauli et al., 1992). The ability of Tat to stimulate expression of TGFβ, a protein that can stimulate a signaling pathway involving the Smad family of transcription factors (Wotton and Massague, 2001), prompted us to investigate transcription of the MCP-1 promoter activity in astrocytes.

In the first series of experiments, we assessed the ability of two important downstream substrates of the TGFβ signaling pathway, i.e., Smad-3 and Smad-4, in regulating transcription of MCP-1 in human astrocytic cells. Results from cotransfection studies revealed that ectopic expression of Smad-3, but not Smad-4, drastically elevates the MCP-1 promoter encompassing the region between −500 and +1 (Fig. 1). Of interest, Smad-4 showed an inhibitory effect on Smad-3-mediated activation of MCP-1 transcription. Progressive removal of the nucleotide sequences between −500 and −100 decreased (more than 50%) the extent of MCP-1 activation by Smad-3, yet maintained the inhibitory effect of Smad-4 on Smad-3. It was also noted that Smad-4 exhibited a less inhibitory effect than Smad-3 on the activation of the MCP-1 promoter containing −100 to +1 in the transfected cells. Altogether, these observations suggested that TGFβ signaling factors such as Smad-3 can up-regulate transcription of the MCP-1 promoter and that

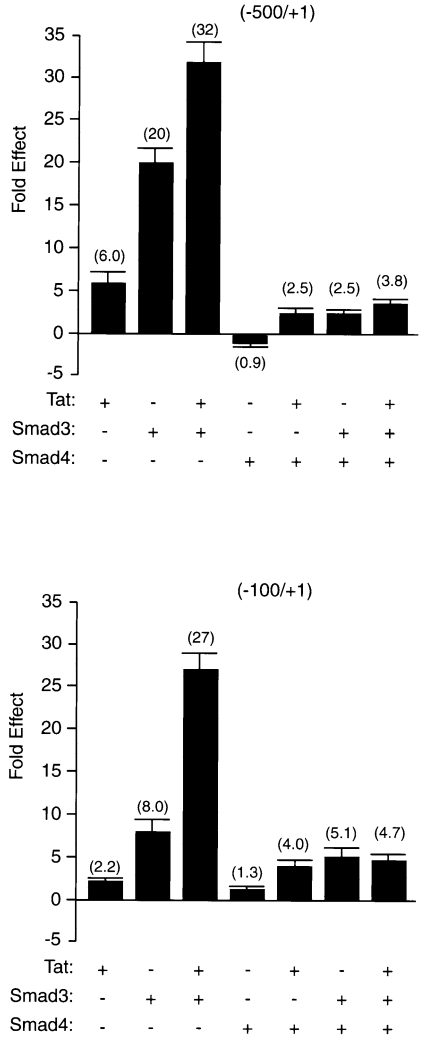

Fig. 2. Effect of Smad-3 and Smad-4 on Tat-induced transcription of the MCP-1 promoter. U-87MG cells were transfected with 5 μg of reporter constructs containing -500 to $+1$ (top) or -100 to $+1$ (bottom) of the MCP-1 promoter along with 5 μg of Tat expression plasmid, 5 μg of Smad-3 expression plasmid, and 5 μg of Smad-4 expression plasmid in various combinations as shown. Fold effect demonstrates the average of three independent experiments.

the intracellular concentration of Smad-3 and Smad-4 can dictate the extent of MCP-1 transcription in astrocytic cells.

In the next series of experiments, we evaluated the cooperative action of Smad-3 and Smad-4 with HIV-1 Tat on transcription of the MCP-1 promoter. As shown in Fig. 2, coproduction of Smad-3 and Tat caused a synergistic activation of the MCP-1 promoter. Again, the observed activation of MCP-1 by Smad-3 and Tat, either alone or together, was drastically decreased in cells overexpressing Smad-4 (Fig. 2A). Similar results were obtained when the MCP-1 minimal promoter sequence ($-100/+1$) was tested in the transfection assay.

As Tat exerts its activity through association with other cellular proteins, we evaluated the possible association of Tat with Smad-3. Results from GST pull-down experiments using protein extracts from U-87MG that overexpressed Smad-3 revealed binding of the Smad-3 to GST-Tat, but not GST (Fig. 3A). To further investigate the specificity of Tat:Smad-3 interaction and determine the region within Tat that can participate in this event, we created a series of deletion mutant Tat proteins in fusion with GST and utilized them in the GST pull- down assay. As illustrated in Fig. 3B, removal of the region between residues 1 and 40 completely abrogated Tat interaction with Smad-3. It was also noted that the mutant peptide containing only the residues between 1 and 50 has a reduced binding activity to Smad-3 compared to the smaller deletions spanning the amino acids between 1 and 40. This observation implies that the residues between 40 and 50 of Tat may modulate its interaction with Smad-3. A similar approach was adapted to study the association of Tat with Smad-4. Results from GST pull-down assay demonstrated that Tat also associates with Smad-4 (Fig 3C) and that the use of Tat deletion mutants of the N-terminus region of Tat indicated that the region spanning residues 1 to 40 is essential for this interaction (Fig. 3D). Interestingly, unlike its interaction with Smad-3, mutant 1 to 50 showed a binding affinity to Smad-4 comparable to that of mutant 1 to 40. As summarized in Fig. 3E, these data show that both Smad-3 and Smad-4 physically associate with Tat and the residues of Tat between 1 and 40 are critical for their interaction.

The Smad family of transcription factors contains common features and is composed of two major domains MH1 and MH2, which are separated by a linker domain (for review, see Wotton and Massague, 2001). As illustrated in Fig. 4A, the MH1 domain of Smad-3 is known for its DNA-binding activity, while MH2 appears to function as the activation domain of Smad-3 and interacts with other DNA-binding proteins.

To gain more information regarding the mechanism for Tat and Smad regulation of the MCP-1 promoter, we created expression plasmids that permit production of two distinct domains of Smad, i.e., MH1 and MH2. Toward this end, PCR-amplified DNA fragments corresponding to MH1 and MH2 were fused to myc Tag DNA fragment and the pCDNA-3 expression plasmid. Production of MH1 and MH2 in U-87MG cells was verified by Western blot analysis using anti-Myc-specific antibody (Fig. 4B). Functionally, expression of the MH1 domain caused a modest induction of the MCP-1 promoter (Fig. 4C). MH2 showed a minimal, if any, stimulatory effect on MCP-1 transcription. Coexpression of MH1 and Tat slightly decreased the ability of Tat to enhance MCP-1 transcription. Under similar conditions, MH2 expression either alone or with MH1 caused a drastic negative effect on the ability of Tat to stimulate MCP-1 transcription. Coproduction of Smad-3 with either MH1 or MH2 alone or together in the cells, abrogated the ability of Smad-3 to stimulate MCP-1 transcription. These observations demonstrate that MH2, and to some extent, MH1 domains of Smad-3 negatively affect the level of activation of the MCP-1 promoter by Tat. Furthermore, both

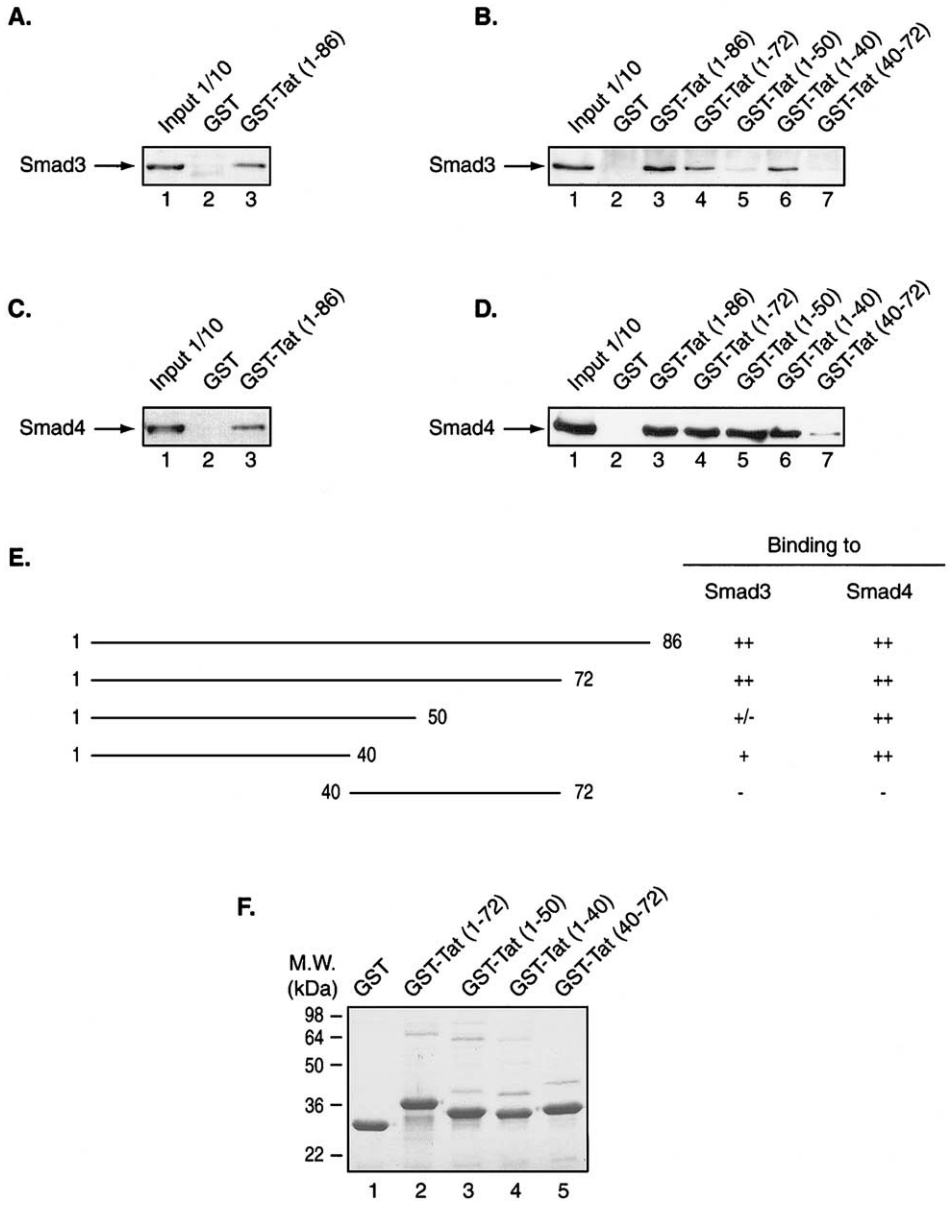

Fig. 3. Interaction of Smad-3 and Smad-4 with Tat protein. Protein extracts from U-87MG cells transfected with plasmids expressing either Smad-3 (A and B) or Smad-4 (C and D) were prepared and utilized in GST pull-down assay according to the procedure described earlier (Gallia et al., 1999). (A) illustrates results from binding of Smad-3 to GST or GST-Tat (1–86) and (B) demonstrates results from binding of Smad-3 to GST and the various mutants of Tat as indicated (Massague, 1998; Sawaya et al., 2000). Lanes 1 in (A) and (B) illustrate the presence of Smad-3 in a 1/10 volume of extract (30 μg) that was used in each GST pull-down assay. (C) and (D) demonstrate binding of GST and GST-Tat (1–86) and Tat mutants to Smad-4. (E) highlights the level of binding of Smad-3 and Smad-4 to the Tat mutant peptides. ++ (strong), + (weak), +/− (very weak), and − (none). (F) illustrates Coomassie blue staining of 10% SDS–PAGE depicting quality of the various Tat proteins that were used in GST pull-down assay.

MH1 and MH2 domains can function as dominant-negative proteins and diminish the level of MCP-1 transcription by Smad-3 in astrocytic cells.

In the next study, we evaluated binding activity of MH1 and MH2 domains to Tat protein. Results shown in Fig. 5 indicate that, similar to full-length Smad-3 protein, MH1 has the ability to associate with Tat and that removal of the region between 40 and 72 decreases, but does not abolish, its binding activity. The Tat mutant protein containing residues 40 to 72 failed to associate with MH1, suggesting that the critical region for MH1 and Tat interaction resides between residues 1 and 40 of Tat. Examination of MH2 interaction with Tat by GST pull-down assay showed a very weak and comparable association of MH2 with the various mutants of Tat, although it was noted that mutant 40 to 72 exhibited a slightly weaker binding activity to MH2 than the other Tat mutants.

In sum, the data presented in this article suggest a potential mechanism for activation of the MCP-1 promoter by HIV-1 Tat through the regulatory proteins that respond to

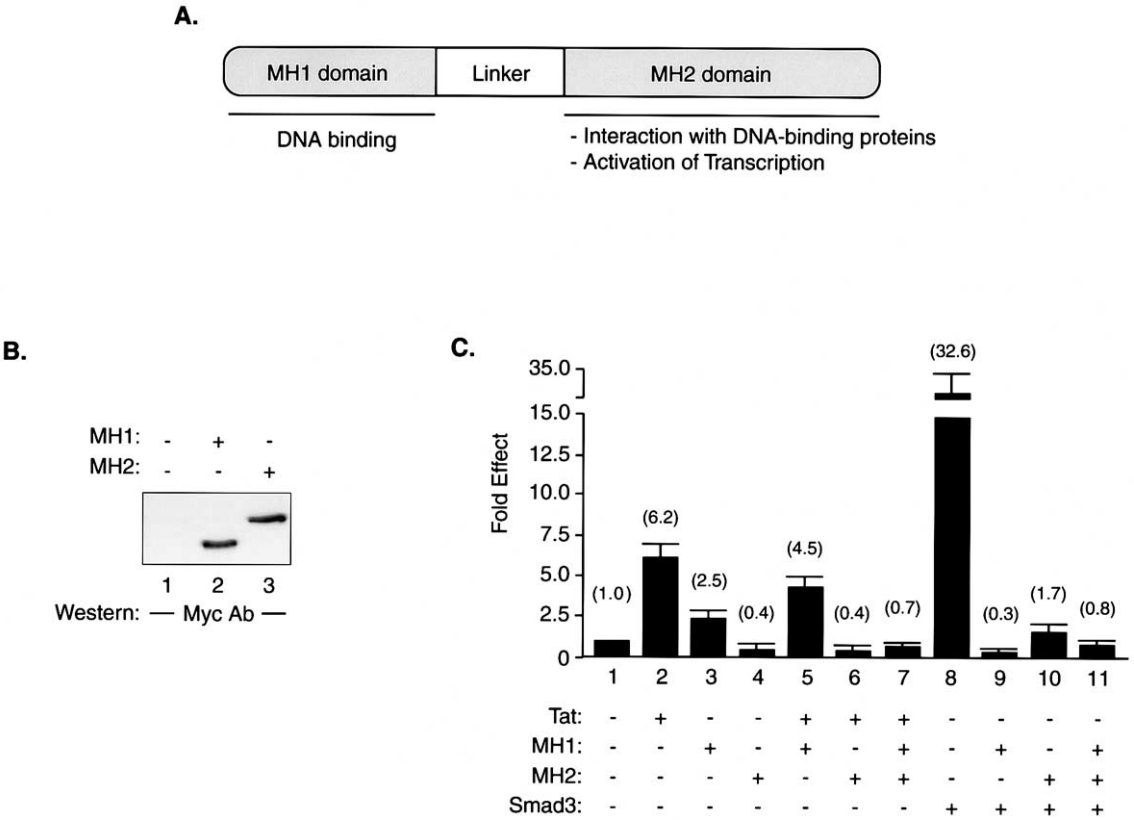

Fig. 4. Effect of MH1 and MH2 on the ability of Tat and Smad-3 in up-regulating the MCP-1 promoter. (A) Schematic representation of Smad-3 structure demonstrating MH1, linker, and MH2 domains. (B) Expression of MH1 and MH2 by the plasmid containing the myc Tag DNA sequence fused to the DNA corresponding to the sequence expressing MH1 and MH2 was determined by Western blot analysis. Human astrocytic U-87MG cells were transfected with 15 μg of a plasmid encoding pCMV-Myc-MH1 and/or MH2. Forty-eight hours posttransfection, cell extracts (50 μg samples) derived from the transfected cells were subjected to Western blot analysis using a Myc-antibody which recognizes the Myc portion in pCMV-Myc-MH1 or MH2 (Santa Cruz Biotechnology, Santa Cruz, CA). The bands corresponding to MH1 and MH2 fusion proteins are shown. (C) U-87MG cells were transfected with 5 μg of MCP-1 reporter constructs (-500 to $+1$) alone or together with plasmids expressing Tat (5 μg), Smad-3 (5 μg), MH1, and MH2 domains (5 μg). All expression plasmids have the same background vector (pcDNA3) and their genes are transcribed by the CMV promoter. The amount of plasmid DNA in each transfection was normalized by empty vector DNA. The values on top of each bar graph represent the average of two separate experiments.

the TGFβ signaling pathway. This is an interesting observation in light of previous data showing that HIV-1 Tat has the ability to stimulate TGFβ gene expression in astrocytic cells (Cupp et al., 1993; Rasty et al., 1996; Sawaya et al., 1998). Thus, one may speculate that Tat activation of TGFβ in astrocytes induces downstream regulators of TGFβ signaling molecules such as Smads that, alone or in cooperation with Tat, modulate expression of MCP-1. In earlier studies it was shown that Tat can also induce expression of tumor necrosis factor-α (TNFα) in various cells and during the course of viral infection of T cells (Buonaguro et al., 1992; Chen et al., 1997; Sawaya et al., 1998). With the notion that both TNFα and TGFβ elevate expression of several chemokines in astrocytic cells (Hurwitz et al., 1995; Oh et al., 1999), one may postulate that the stimulatory effect of Tat on expression of chemokines including MCP-1 can be indirect and involve signaling pathways such as TNFα and TGFβ (New et al., 1997; Sawaya et al., 1998). In support of this notion, earlier studies indicated that binding of the NF-κB transcription factors, whose activity is induced in response to TNFα, to the MCP promoter DNA sequence is increased upon Tat treatment of human astrocytic cells (Lim and Garzino-Demo, 2000).

Our data add Smads to the list of cellular proteins that physically and functionally interact with Tat. Earlier studies on the mechanism of Tat activation of the HIV-1 promoter showed that interaction of Tat with TAR RNA of the LTR on one hand, and cyclin T and cdk9 on the other hand, results in the positioning of cyclin T and cdk9 in close proximity to the transcription start site where they phosphorylate carboxyl terminal domain of RNA polymerase II (Richter et al., 2002; Zhou and Rana, 2002). Other studies have revealed that association of Tat with several other cellular proteins with DNA-binding activity enables Tat to stimulate transcription of genes with no TAR sequence (Ansari et al., 1999; Darbinian et al., 2001; Gallia et al., 1999; Jeang et al., 1993; Taylor et al., 1994). For example, the association of Tat with Purα can result in the activation of the JC virus and TGFβ promoters in glial cells (Krachmarov et al., 1996; Thatikunta et al., 1997). Similarly, the

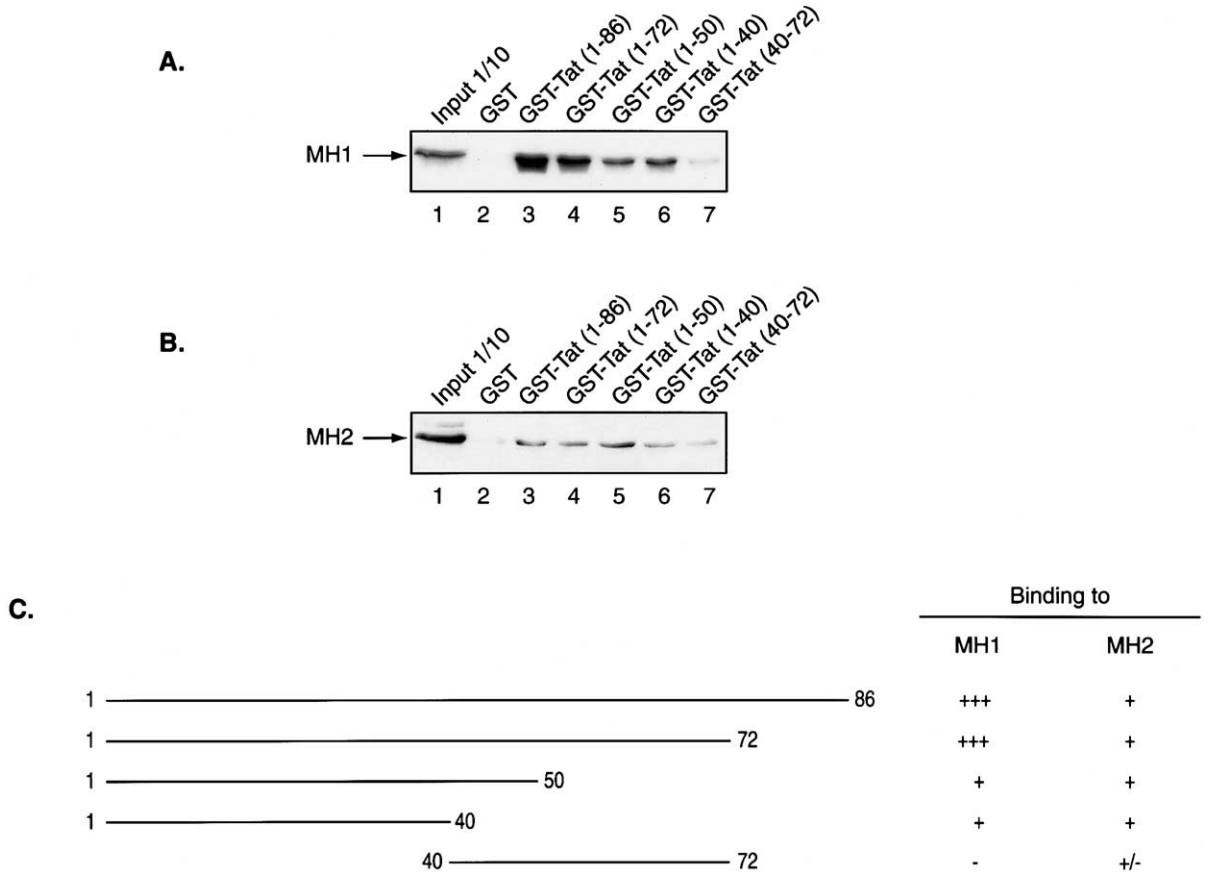

Fig. 5. Interaction of MH1 and MH2 with Tat. Protein extracts from U-87MG cells transfected with 50 μg of pcDNA3-MH1 (A) and pcDNA3-MH2 (B) were prepared and mixed with GST, GST-Tat, or various Tat mutants. (A) Proteins bound to GST, GST-Tat, or GST-Tat mutants were analyzed by Western blot using Myc-specific antibody. (C) The intensity of the band corresponding to MH1 and MH2 (shown in A and B), respectively, was densitometrically determined and rated as $+++$ (strong), $+$ (weak), and $+/-$ (very weak), and $-$ (none).

interaction of Tat with the NF-κB subunits or SP1 under certain physiologic conditions enables Tat to stimulate TAR-negative HIV-1 LTR transcription (Taylor et al., 1994; Widlak et al., 1997). The cooperative interaction of Tat with Smad-3 upon the MCP-1 promoter indicates that under these conditions, when the TGFβ signaling pathway is at work, expression of MCP-1 gene in astrocytes can be affected by Smads. Further, our results reveal new peptides, MH1 and MH2 that can be utilized as inhibitory compounds to block activation of MCP-1 by Tat. Currently, studies are in progress to assess the ability of MH1 and MH2 in suppressing MCP-1 expression in response to TGFβ stimulation in several other cell types as well as in an in vitro system.

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